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# Phosphorylation of basic fibroblast growth factor (FGF-2) in the nuclei of SK-Hep-1 cells

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The subcellular fractions containing protein kinases capable of phosphorylating basic fibroblast growth factor (FGF-2) are unknown, but having previously characterized one that is associated with the plasma membrane [1991, Mol. Endocrinol. 5, 1003–1012] we evaluated the catalytic properties of another in the nucleus. The reaction is time (linear up to 15 min), enzyme (2,000–25,000 nuclei/ml), and substrate ($K_m$ 0.18 $\mu$M) dependent, and the targets serine. DNase pretreatment of nuclei decreases the incorporation of phosphate into FGF-2 by 50% and the reaction. It is also inhibited by heparin ($EC_{50}$ 1 $\mu$g/ml) and spermidine ($EC_{50}$ 3 $\mu$M). Calcium and cAMP have no effect. We conclude that the kinase is distinct from PKA, and PKC, and suggest that changes in glycosaminoglycan and polyamine concentrations during the cell cycle may modulate FGF-2 phosphorylation in the nucleus, or as it is translocated to the nucleus.

Growth factor; Nucleus; Kinase; Phosphorylation; FGF; SK-Hep cell

## 1. INTRODUCTION

Basic fibroblast growth factor (FGF-2) is a potent mitogen and morphogen for many diverse cell types [1,2]. In cell culture, it is synthesized by numerous cells, including endothelial cells [3,4], granulosa cells [5], and various types of tumor cells [6–8]. Over 95% of the growth factor produced by these cells, however, remains cell associated, since the mature protein lacks a signal sequence to mediate secretion [9]. Thus, although basic FGF appears to be exported to the cell surface [10] and sometimes deposited into the extracellular matrix [4,11], the mechanism for its translocation to and from the matrix is unknown.

Attempts to understand the regulation of FGF-2 have been complicated by recent observations showing its localization in the cytoplasm and nucleus of numerous cell types [10,12–15]. Because FGF-2 can be phosphorylated [16,17], we have proposed this post-translational change might regulate its potential paracrine, autocrine, and even intracrine activities [17,18]. The phosphorylation and dephosphorylation of proteins has long been recognized as an important regulatory mechanism of cellular function [19,20]. Protein kinases exist in a variety of enzymatic forms with distinct subcellular distributions and various substrate specificities. In the study here, we describe the presence of a nuclear, serine-specific FGF-2 kinase which is detectable in the nuclei of a cell type that synthesizes FGF-2, SK-Hep-1 cells. In contrast, this activity is not readily detectable in the nuclei of cells (Balb-C 3T3 fibroblasts) that do not synthesize the growth factor.

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## 2. MATERIALS AND METHODS

### 2.1. *Materials*

cAMP, and PK-I (a heat- and acid-stable inhibitor of cAMP-dependent protein kinase), spermidine, heparin and DNase were purchased from Sigma. [$\gamma$-$^{32}$P]ATP (3,000 Ci/mol) was from ICN Radiochemicals (Irvine, CA). Reagents for polyacrylamide electrophoresis (PAGE) were obtained from Bio-Rad. Recombinant human FGF-2 was a generous gift of Dr. Paolo Sarmientos, Farmitalia Carlo Erba, Milano, Italy and the antiserum to FGF-2 has been described elsewhere [21].

### 2.2. *Cell cultures and immunohistochemistry*

Human hepatoma cells (SK-Hep-1) and Balb-C 3T3 fibroblasts were obtained from the ATTC repository, grown as monolayers in DME/MEM containing Earle's salts supplemented with 10% (v/v) calf serum (Flow Laboratories, Irvine) and kept in humidified air/$CO_2$ (95:5; 37°C) atmosphere. The cells were prepared for immunohistochemistry as described by Florkiewicz et al. [10]. Specificity of staining was determined using a preparation of antibody whose anti-FGF IgG had been preadsorbed to an FGF-2 affinity column.

### 2.3. *Preparation of purified nuclei*

The technique used to prepare nuclei was developed from that described elsewhere [28]. SK-Hep-1 cells were grown in 10 cm dishes, and 30 dishes were routinely used for the preparation of nuclei. The cells were washed 3 times with cold phosphate-buffered saline (PBS), scraped in 50 ml PBS, and centrifuged at 3,000 rpm. The cell pellet was homogenized in 10 ml of a HSKM buffer (pH 7.4) that consists of HEPES (25 mM), 0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, 0.5% Triton X-100 and protease inhibitors (50 $\mu$g/ml leupeptin, 1 $\mu$g/ml aprotinin, 1 mM EDTA, and 10 mg/ml pepstatin). The cell homogenate was incubated at 4°C for 15 min and centrifuged for 5 min at 3,000 rpm. The pellet was gently resuspended with a Teflon pestle and rinsed 3 times with 5 ml of HSKM buffer (without Triton). The final


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pellet containing purified nuclei was used in the kinase assay or stored at −80°C. The original supernatant was further centrifuged at 125,000 × *g* for 45 min to obtain plasma membranes, and the supernatant was considered the cytosolic fraction. Balb-C 3T3 cells were processed in a similar fashion to obtain plasma membranes, cytosol, and nuclei.

2.4. *Extractions and Western blotting*

Nuclei were extracted in a solution of 1% NP-40, 0.5% deoxycholate, 1 mM PMSF, 1 mM EGTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 2 M NaCl in 2 mM Tris-Cl, pH 7.4, as described by Florkiewicz and Sommer [22]. The extract was centrifuged and the supernatant was either analyzed directly or diluted to a conductivity <0.2 M NaCl. In these latter samples, an aliquot (50 μl) of heparin–Sepharose was added to the solution and incubated at 4°C. This solution was centrifuged, the heparin–Sepharose beads washed with 0.6 M NaCl, and the proteins bound were solubilized with Laemmli's sample buffer [23] and loaded onto a 15% acrylamide/0.1% bisacrylamide gels and electrophoresed. FGF-2 was visualized by Western blotting using an antibody to FGF-2 as described [24].

2.5. *Phosphorylation assays*

The indicated concentrations of nuclei were incubated with FGF-2 or the indicated substrates in a final volume of 40 μl. The reaction was started by the addition of a reactive solution containing [$\gamma$-$^{32}$P]ATP (5 μM, 1 μCi/assay) and 5 mM $MgCl_2$. At the end of the incubation, 10 μl of a 5× Laemmli loading buffer was added to stop the reaction. The samples were boiled and phosphorylated proteins were analyzed by autoradiography after SDS-PAGE.

2.6. *Phosphoamino acid analyses*

Phosphorylated peptides and proteins were prepared for phosphoamino acid analyses as described by Cooper et al. [25]. After extraction from the dried gel, the phosphoproteins were hydrolyzed in 6 N HCl for 90 min at 110°C. The phosphoamino acids were separated by high voltage electrophoresis on cellulose plates at pH 1.9 and pH 3.5, and the separation visualized by autoradiography.

## 3. RESULTS

3.1. *Distribution of FGF-2 in SK-Hep-1 cells*

As shown in Fig. 1A, an immunoreactive FGF-2 (ir-FGF-2) is associated with the cell surface when the cells are not permeabilized. Under permeabilizing conditions, most of the staining is found in the nucleus (Fig. 1B). This localization is not exclusive, as there is faint, but significant, staining in the cytoplasm. Immunoelectron microscopy shows this cytoplasmic staining in the perinuclear region of SK-Hep-1 cells (not shown).

In an effort to identify the molecular forms of the ir-FGF-2 in the nuclei, nuclear proteins were extracted and processed for Western blotting. As shown in Fig. 1C, the antibody to FGF-2 detects the 18, 22, and 24 kDa molecular forms of FGF-2 [22] which bind to immobilized heparin.

3.2. *Endogenous protein kinases in SK-Hep-1 cells*

The subcellular fractions from SK-Hep-1 cells were prepared as described in section 2, used as potential sources of protein kinases and tested for their ability to phosphorylate FGF-2. Briefly, [$\gamma$-$^{32}$P]ATP (5 μM, 1 μCi/assay) and $MgCl_2$ (5 mM) were added to aliquots of cytosol, plasma membranes, or nuclei alone or in combination with 1 μg of FGF-2 as substrate. After a 15 min incubation at room temperature, the reaction was terminated by the addition of Laemmli's sample buffer, and the phosphorylated proteins were analyzed by SDS-PAGE and autoradiography. Total protein was visualized by Coomassie blue staining. As shown in Fig. 2 at the concentration tested, the cytosolic fraction of SK-Hep-1 cells contains no detectable FGF-2 kinase activity, while the plasma membranes and nuclei preparations of SK-Hep-1 cells (Fig. 2B) have a significant ability to phosphorylate FGF-2. The reaction is particularly impressive in nuclei, since so little protein is present in the reaction (Fig. 2A). Identical incubations with subcellular fractions of 3T3 cells reveal that these cell compartments contain barely detectable levels of FGF kinase activity (Fig. 2C,D).

3.3. *Kinetic properties of nuclear FGF-2 kinase*

Having previously described an ecto-protein kinase activity that is associated with the plasma membrane of SK-Hep-1 cells, we focused further studies on the phosphorylation of FGF-2 by nuclear protein kinase(s). The kinetic properties of the enzyme were examined by modifying the effects of time, concentration of enzyme (nuclei), and substrate. When the number of purified nuclei is increased from 2,500/ml to 85,000 nuclei/ml, there is a dose-dependent increase in the phosphorylation of FGF-2 (Fig. 3A). The reaction is time dependent, and the incorporation of phosphate into FGF-2 is linear for 10 min at 22°C, with up to 100,000 nuclei/ml (Fig. 3B). The substrate specificity of the nuclear kinase was determined using histone H2A, casein, and FGF-2 as exogenous substrates. The enzyme preparation preferentially phosphorylates FGF-2 → casein → histone (not shown), and Lineweaver–Burke analyses of the dose curves approximate an apparent $K_m$ of 0.18 μM. This value is well within the expected range for a potentially physiological substrate. In these crude preparations of enzyme however, the stoichiometry is low (<0.3 mol phosphate/mol FGF-2) but increases with purification (unpublished observations). Phosphoamino acid analyses reveal that the enzyme targets serine, thus classifying the enzyme as a serine kinase (not shown). Mapping of this site is in progress.

3.4. *Characterization of the phosphorylation of FGF-2 by purified nuclei*

Our initial attempts to characterize the phosphorylation of FGF-2 by purified nuclei are presented in Table I. The presence of magnesium is required for nuclei to phosphorylate FGF-2. While this activity is increased by concentrations of $Mg^{2+}$ below 10 mM, higher concentrations are inhibitory. For this reason, all subsequent assays for the enzyme were performed in the presence of 5 mM $Mg^{2+}$. $Ca^{2+}$ cannot substitute for $Mg^{2+}$, and there is no additive effect when both ions are present in the assay buffer. Agents that modulate the activ-

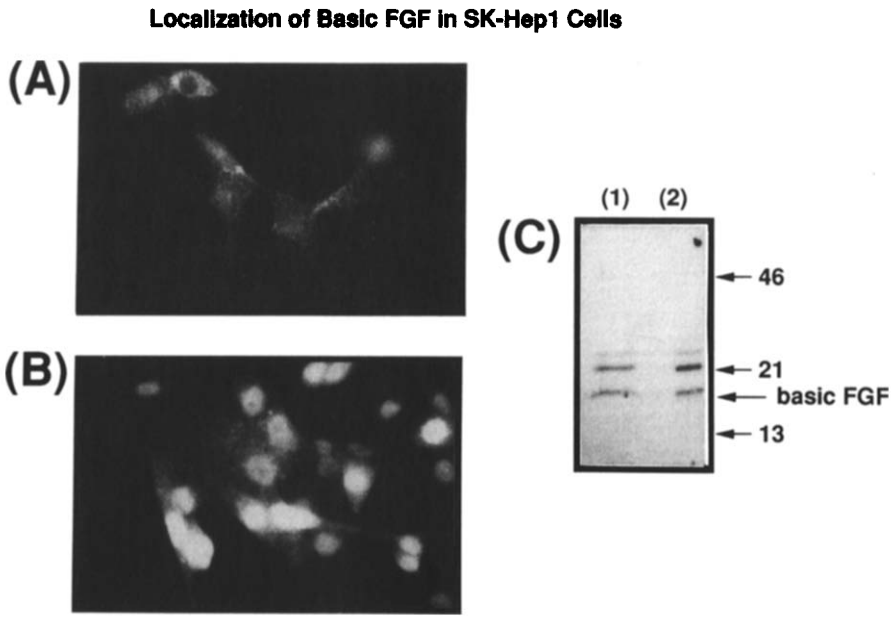


Fig. 1. Localization of FGF-2 in SK-Hep-1-1 cells. Cells were grown in culture as described in the text, and the presence of FGF-2 established by immunohistochemistry of nonpermeabilized (Panel A) and permeabilized (Panel B) cells. The antigens recognized are the forms of FGF-2s that are expected as determined by Western blotting of the extracts without (1) or with (2) adsorption and elution from heparin-Sepharose (Panel C). The molecular weight markers ($\times 10^{-3}$) and mobility of FGF-2 are indicated.

ities of protein kinase A (PK-A) or protein kinase C (PK-C) have no effect on the phosphorylation of FGF-2 by the protein kinase. Neither cAMP, the heat- and acid-stable PK-A inhibitory protein PK-I, phorbol esters, or a mixture of phosphoatidyl serine/dolein changes the phosphorylation pattern of FGF-2. When the assays are performed in the presence of increasing concentrations of spermidine or heparin, there is a dose-dependent inhibition of phosphorylation of FGF-2 by the purified nuclei. Under the assay conditions used here, spermidine had an $IC_{50}$ of 3 mM, while heparin inhibits at 1 $\mu$g/ml. Accordingly, the reaction appears to be sensitive to an effect of glycosaminoglycans and polyamines which are presumably acting either on

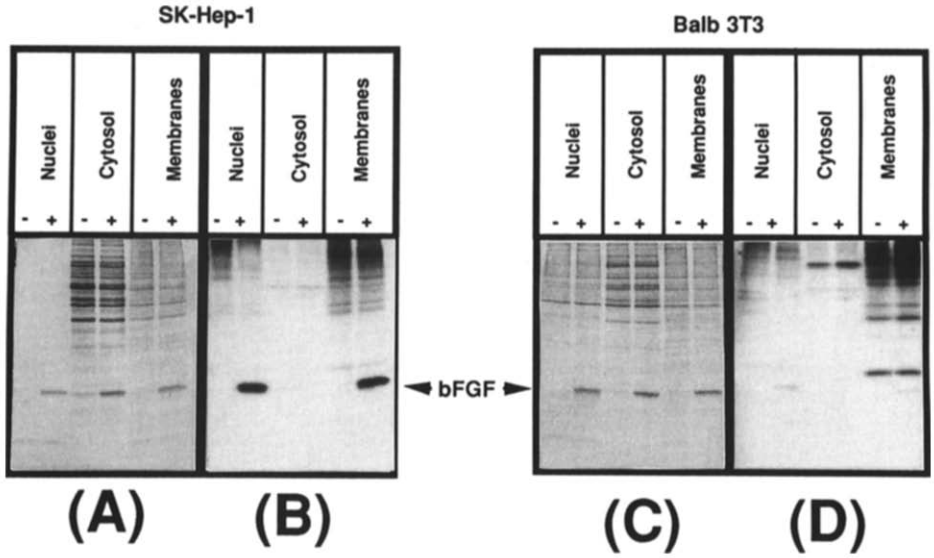


Fig. 2. Subcellular localization of FGF-2 kinase in SK-Hep-1 and Balb-C 3T3 cells. Subcellular fractions from SK-Hep-1 (A,B) and Balb-C 3T3 cells (C,D) were obtained as described in section 2. An aliquot of each fraction (15 $\mu$l) was incubated with (+) or without FGF-2 (1 $\mu$g/ml) and [$\gamma$-$^{32}$P]ATP for 20 min at room temperature. The reaction was stopped by addition of a 5× solution of Laemmli sample buffer (5 $\mu$l). Proteins were analyzed by SDS-PAGE and visualized by Coomassie blue staining of the gels (Panels A and C), and the extent of phosphorylation by autoradiography (Panels B and D). The mobility of FGF-2 is indicated.

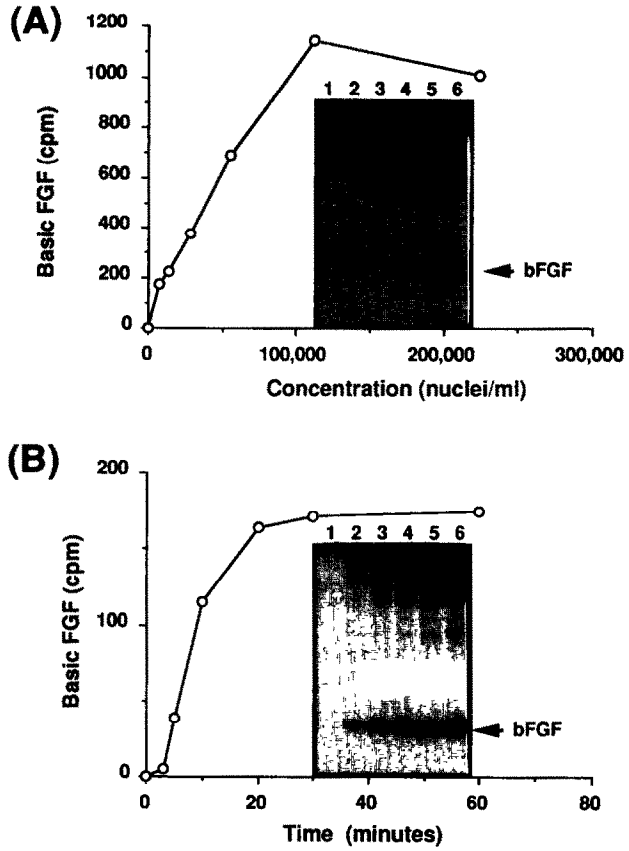


Fig. 3. Kinetics of the phosphorylation of FGF-2 by the purified nuclei of SK-Hep-1 cells. FGF-2 (500 ng/tube) was phosphorylated by increasing concentrations of nuclei (Panel 3A) as described in the text, and the radiolabelled FGF-2 examined by autoradiography after SDS-PAGE. The FGF-2 shown in the inset were cut from the gel and counted on a $\beta$-counter to yield the data shown in the graph. The phosphorylation assay was also performed with a fixed number of nuclei, but stopped at different times (Panel B). The bands in Lanes 1 through 6 correspond to incubations stopped at the indicated times, respectively. The FGF-2 shown in the inset were cut from the gel and counted on a $\beta$-counter to yield the data shown in the graph.

FGF-2 itself to prevent its association with the nucleus, its interaction with the kinase, or by interacting with the kinase directly. Each of these possibilities is currently being investigated with the purified enzyme.

## 4. DISCUSSION

The findings presented here demonstrate the existence of protein kinases distinct from wither PKC or PKA which can phosphorylate FGF-2. They are present in the plasma membranes and nuclei of proliferating SK-Hep-1 cells. Only a small component of these activities might be attributable to the presence of the known kinases that can phosphorylate FGF-2 like PK-C or PK-A [29,30], because the major activity in each compartment appears distinct. In previous studies [17,26], we characterized a membrane-bound ectoprotein kinase that phosphorylates FGF-2, and we presume the activities detected here in the plasma membrane preparations are due to this same enzyme. The nuclear kinase activity, however, appears novel. It is not cAMP, phospholipid, and calcium dependent (manuscript in preparation), and is barely detectable in proliferating Balb-C 3T3 cells. Balb-C 3T3 is a cell type which has no endogenous FGF-2, but responds well to the growth factor.

Human hepatoma SK-Hep-1 cells synthesize large quantities of FGF-2 and possess high affinity receptors, yet respond poorly to exogenous FGF-2. Although its intracellular localization is essentially nuclear, the possible role (if any) of FGF-2 phosphorylation in the nucleus is not known. For several years now, the importance of phosphorylation in the activation of DNA-binding proteins has been described for transcription factors. In view of FGF-2 being localized in this compartment, it may be worth considering the possibility that it is itself a transcription factor and/or a *cis*- or *trans*-acting element regulated by phosphorylation. Thus FGF-2 resembles c-*fos* [31], CREs [32], and serum responsive elements [33] to the extent it localizes to the nucleus [27], is phosphorylated [29,30], and its subcellular distribution appears linked to the cell cycle, cell growth, and differentiation [27,39]. Of course, the phosphorylation of FGF-2 could also play a role in its trafficking through different compartments of the cell [36–38]. Accordingly, it is particularly interesting to note the abundance of the nuclear FGF-2 kinase activity in the

Table I

Characterization of the phosphorylation of FGF-2 by purified nuclei

| Treatment | Concentration | [$^{32}$P]FGF (cpm) |
|---|---|---|
| $MgCl_2$ (mM) | 0.3 | 56 |
| | 2.0 | 294 |
| | 5.0 | 322 |
| | 10.0 | 634 |
| | 50.0 | 297 |
| Control | (+10 mM $MgCl_2$) | 608 |
| $CaCl_2$ | 0.45 mM | 549 |
| cAMP | 3.5 mM | 511 |
| cAMP + PKI | 3.5 $\mu$M | 622 |
| Heparin ($\mu$g/ml) | 0.0 | 608 |
| | 0.4 | 469 |
| | 0.8 | 414 |
| | 3.2 | 142 |
| | 12.5 | 17 |
| | 31.2 | 18 |
| Spermidine (mM) | 0.0 | 622 |
| | 0.37 | 519 |
| | 0.73 | 498 |
| | 1.46 | 684 |
| | 2.90 | 419 |
| | 5.85 | 420 |
| | 11.70 | 220 |

cells which have endogenous FGF (SK-Hep-1 cells), compared those that do not (Balb-C 3T3 cells).

Finally, it is important to highlight the potent inhibitory effects of both heparin and spermidine on the phosphorylation of FGF-2 by nuclei. Both molecules belong to families which have critically important roles in the control of normal cell growth and function. For example, heparin can potently suppress the entry of cells into S-phase in response to mitogens by arresting them early in the cell cycle, and can selectively inhibit the mitogenic stimulation of cells by TPA and serum. Polyamines as well have long been associated with cell proliferation and differentiation. In view of the striking effects of both heparin and spermidine on the phosphorylation of FGF-2 by the purified nuclei, it is interesting to speculate that these agents might also regulate FGF-2's phosphorylation in vivo. If so, a trophic stimulus modulating the intracellular levels of GAGs and polyamines would, in turn, modulate the nuclear FGF-2 phosphorylation. Whether this effect is directly on the kinase or mediated through other components of the nuclei (e.g., transport), these observations further support a potential role for FGF-2 phosphorylation in the regulation of cell growth and function.

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